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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

This grant is a Career Development Award. Since this very first funding award to the P.I., I have successfully obtained some other grants, either serving as the P.I. or Investigator. The training experience was very important for me to become a competitive investigator. MR imaging was applied to longitudinally monitor the tumor volumetric changes as well as the vascular changes. The histological examination technique was developed to quantitatively measure the vascular area from the H&E staining tumor slides. We also assessed the degree of inflammation in the tumor. In Yr-03 we studied 48 tumors with MRI. The baseline study was first performed, then the adenoviruses carrying different genes were injected intratumorally. Five days later the follow-up MRI study was performed to determine the growth rates. The tumors were then excised for histological analysis to determine the vascular area and the inflammation scale. We found that the MRI volumetric or vascular properties could not reliably predict the tumor growth, however, it can be used as a suitable monitoring modality. We further attempted to correlate the MRI findings with the underlying biological changes, including vascularity and inflammation. However, no significant correlation was found. Nevertheless, the techniques that were developed in this project can be applied to other projects in the future.

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(4) Introduction

This training proposal (Career Development Award) has two major goals: 1) Personally for me to go through the necessary training and become an independent breast cancer investigator, and 2) Scientifically to find the non-invasively measurable imaging parameters that reflect the underlying biological changes to predict the eventual efficacy of gene therapy. For the personal training, I have taken some courses in tumor biology and Virology in Yr-01. For the scientific objectives, I planned to choose three appropriate recombinant viral systems to work with. Ideally the three viral systems should work through the mechanisms of direct cell killing, indirect killing via microvasculature damage, and a combination of both, respectively, in achieving the therapeutic effect. However, the attempt was not easy to achieve. Although the concept of anti-angiogenic gene therapy has been proposed and some preliminary success has been accomplished, it is not easy to obtain these virus systems. The Vector Center at UC Irvine did not have the resources to develop our own anti-angiogenic vector. Therefore, I chose to use three available systems: adenovirus carrying interleukin-1 alpha, interferon-gamma, transforming growth factor–beta, and beta gal as control. MR imaging was applied to longitudinally monitor the tumor volumetric changes as well as the vascular changes. We have also developed histological tissue examination techniques to quantitatively measure the vascular area from the H&E staining tumor slides. We also assessed the degree of inflammation in the tumor. In Yr-03 we studied 48 tumors with MRI. The baseline study was first performed, then the adenoviruses carrying different genes were injected intratumorally. Five days later the follow-up MRI study was performed. The growth rates of these tumors were determined from the baseline and follow-up studies. The tumors were then excised for histological analysis to determine the vascular area and the inflammation scale. The results obtained from the imaging study were correlated with the underlying biology to investigate their relationships. The detailed results are summarized in the body of this report.

(5) Body

In the proposal I planned to achieve 6 specific aims during the three year funding period:

*** 1. Obtain basic training in general tumor biology and standard laboratory work,

*** 2. Pursue specific training in the application of recombinant virus technology for gene therapy,

* 3. Choose and, if necessary, develop three appropriate recombinant viral systems that are expected to cause direct cell killing, indirect killing via microvasculature damage, and a combination of both, respectively,

** 4. Study the longitudinal structural and vascular changes in tumors receiving the three viruses with MRI,

** 5. Study the associated changes in the biological or pathological characteristics of the tumors at various times after the treatment,

** 6. Correlate the changes in the MRI parameters with the underlying biological changes to establish the relationships between them.

"***" completed in Yr-01, "**" completed in Yr-02-03, "*" could not be achieved

I have been working on Aims 4-6 during Yr-03 of the project period. For Aim 3 I have been working on searching the appropriate systems for the proposed study, i.e. to find genes that can be encoded into adenovirus to reach therapeutic effect by direct cell killing and inhibition of angiogenesis. I have done extensive literature search. The search results have been summarized in Yr-01 report. I have also obtained a plasmid containing the VEGF 121 gene (Vascular Endothelial Growth Factor), from Dr. A. Harris at the University of Oxford, UK. Initially we were planning to transfect the gene into adenovirus. However, this requires a great effort. The Vector Center at our institute did not have the available resources to carry out this difficult task. It also requires a great financial support, which is not supported by this training grant. Therefore, Aim 3 could not be achieved. Instead, we chose to use three available adenovirus systems provided by our Vector Center.

We focused on using imaging techniques to monitor the changes taking place in tumors after being infected with adenovirus carrying different cytokines. The genes which had been studied included mouse interferon γ (IFN-γ), human transforming growth factor β (TGF-β), and mouse interleukin 1-α (IL1-α), or the marker gene β-gal. The relationship between the results obtained by in-vivo imaging and the underlying changes measured by histological analysis was investigated. The methods and the results are summarized in this section.

Methods:

Tumor Model:

C6 glioma cells implanted subcutaneously and bilaterally into the rear haunch of Wistar rats (5 million cells were injected). Two weeks after the injection when the tumors reached to at least 0.7 cm in diameter, the baseline MRI study was performed.

Preparation of Recombinant Adenovirus:

A. Purification of Plasmid DNA
B. DNA Transfections for Rescue of Recombinant Adenovirus Vectors: Using Lipofectamine methods
C. Amplification of a Clonal Viral Stock
D. Extraction of Viral DNA from 293 cells
E. Titration, Large Scale Propagation and Purification of Adenovirus

Adenovirus encoding:
mouse interferon gamma (IFN-γ)
mouse interleukin 1 alpha (IL 1-α)
human transforming growth factor beta (TGF-β)
marker gene (β-gal)

Gene Expression of C6 glioma cells to the virus system:
The in-vitro and in-vivo expression of β-galactosidase were confirmed.
In-vitro experiments showed that corresponding to the MOI (multiplicity of infection) of 10, 1, and 0.1, 80%, 50%, and 5% of the cultured cells expressed the recombinant b-gal gene. The vivo experiments showed that the recombinant gene expression was localized at the site of viral injection.

Treatment:

One day after the baseline MRI study, animals received right-side intratumoral injection of 2x10^8 plaque forming units of virus.

MRI Studies:

1. Interleaved T2-weighted images (fast SE) across the tumor for measurement of the total tumor volume.
2. Select 2-3 slices covering the tumor for the dynamic study. Also select a slice through the liver to measure the liver kinetics.

3. The dynamic study. Inject 0.1 mmol/kg Gd-DTPA after 4 pre-contrast acquisition.
4. Wait at least 1 hour for the clearance of Gd-DTPA and start another dynamic study. This time, inject 0.05 mmol/kg Gadomer-17.
5. The MRI studies were repeated at 5 days after the treatment.

Histological studies for quantitative vascular area measurement:

All studies were performed on tissues that were fixed in neutral buffered formalin and embedded into paraffin blocks in the usual manner. Sections of the tumors were cut at 5 micron thickness and then stained with hematoxylin and eosin prior to analysis. Each tumor was evaluated independently by two pathologists who recorded ten digital images at 40x power from each slide using a *Spot* digital camera (Diagnostic Instruments Inc., Sterling Heights, MI) and a Nikon Eclipse E600 microscope. A typical slide included two to four different sections of the tumor. Each image represented a randomly selected, non-overlapping region of tumor and connective tissue. The individual images were then digitally dissected and analyzed using the *Image-Pro Plus version 4* image analysis software (Media Cybernetics, Silver Spring, MD). In order to select blood vessels for measurement, we used additive 3x3 pixel cubes of defined RGB value. The RGB color values were defined by the color of red blood cells in the image and by the color of the empty space in the blood vessels. Generally each image required at least three cube values for assessment. After the operator defined the RGB values and the area of interest, the statistical program accompanying the image analysis software then counted the number of pixels with the defined RGB values within the defined area to calculate the percentage of the vascular area. An example for the digital dissection of blood vessels in a representative section of a tumor in a rat is shown in Figure 1.

Assessment of Inflammation:

Each slide was examined for the presence of lymphocytes and plasma cells (chronic inflammation) and mast cells. The grading scale was defined as 0=absent, 1=present, 2=abundant. If inflammatory cells appeared to constitute less than 5% of the total cells, the case was graded as "1". All cases had at least some chronic inflammation. If the lymphocytes and plasma cells were more than 5% of the total cells, the specimen was graded as "2". For mast cells, tissue sections with 5 or fewer intact mast cells were graded as "1". More than 5 mast cells total was graded as "2".

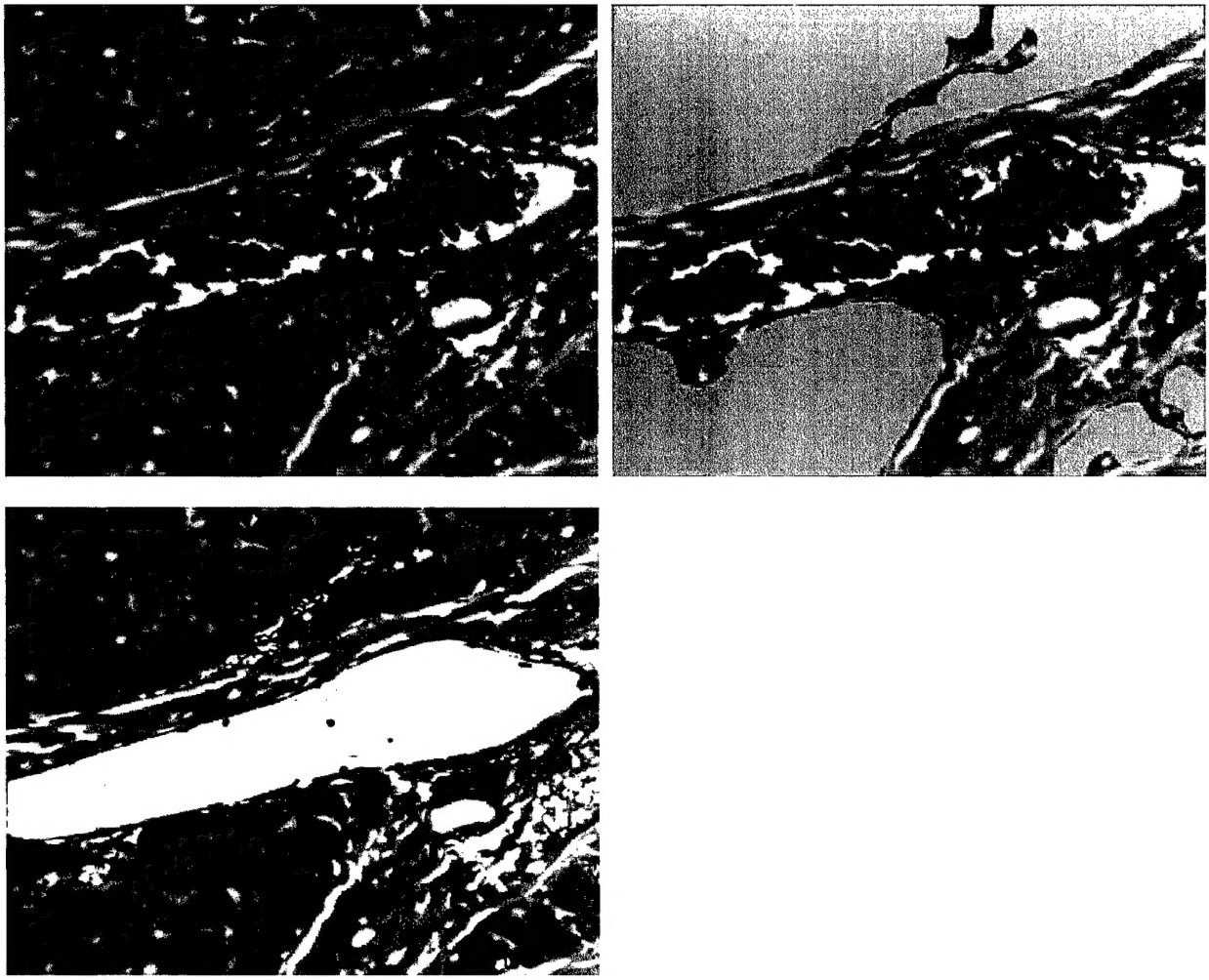

Figure 1: Digital dissection of blood vessels in a representative section of a tumor in a rat. The region of interest was selected by manually deleting the portions of the image that were occupied by tumor cells (green, in b) or necrosis. After the RGB values for red blood cells and lumenal spaces were defined by the operator, the software automatically selected the blood vessels within the image (yellow, in c) and counted the number of pixels within the selected region.

Results:

The study protocol was applied to a group of 24 rats with bilateral tumors. The baseline and follow-up studies were successfully carried out from 38 tumors in 19 rats. The right side of tumor was treated with adenovirus carrying IL-1α, IFN-γ, or β-gal gene. The volumetric growth rates between baseline and follow-up studies and the contrast enhancement kinetics were measured. According to their growth rates (the volume at follow-up /the volume at baseline), the tumors were categorized into four groups:

Group-1 (n=11): Growth Ratio < 1
Group-2 (n=12): 1< Growth Ratio < 2.5
Group-3 (n=10): 2.5 < Growth ratio < 3.5
Group-4 (n=5): Growth ratio > 3.5

We were interested in studying whether the baseline tumor size or baseline vascular volume or the changes of vascular volume can predict their future growth, i.e. to distinguish these 4 groups with significant differences. Figure 2 shows the mean and the standard deviation of the baseline tumor volumes in the 4 groups. There was a wide variation in groups 1 and 2. The baseline volume did not reveal significant differences among these 4 groups.

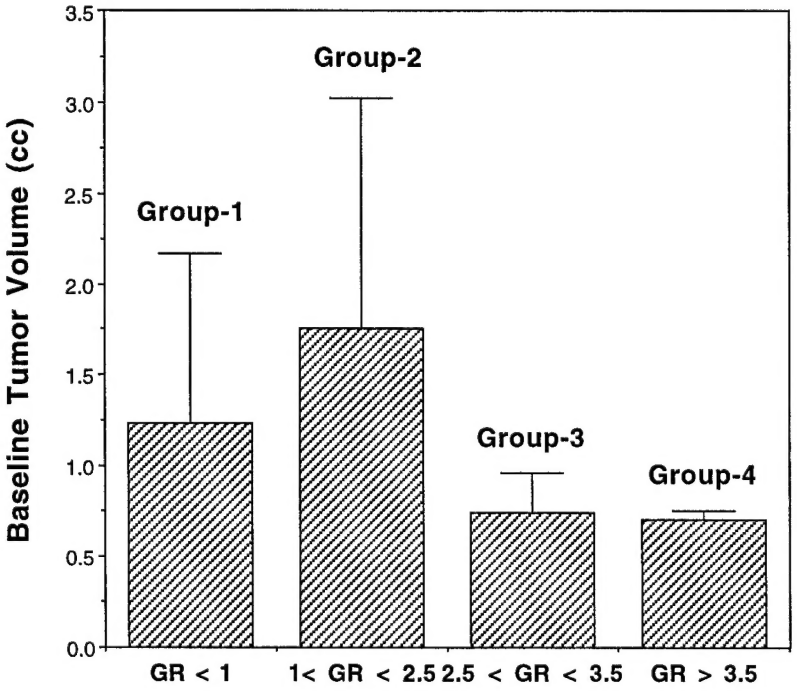

Figure 2: The baseline volume of tumors in these 4 groups. The tumors in Groups 1 and 2 had large variation. They did not reveal significant differences among these 4 groups.

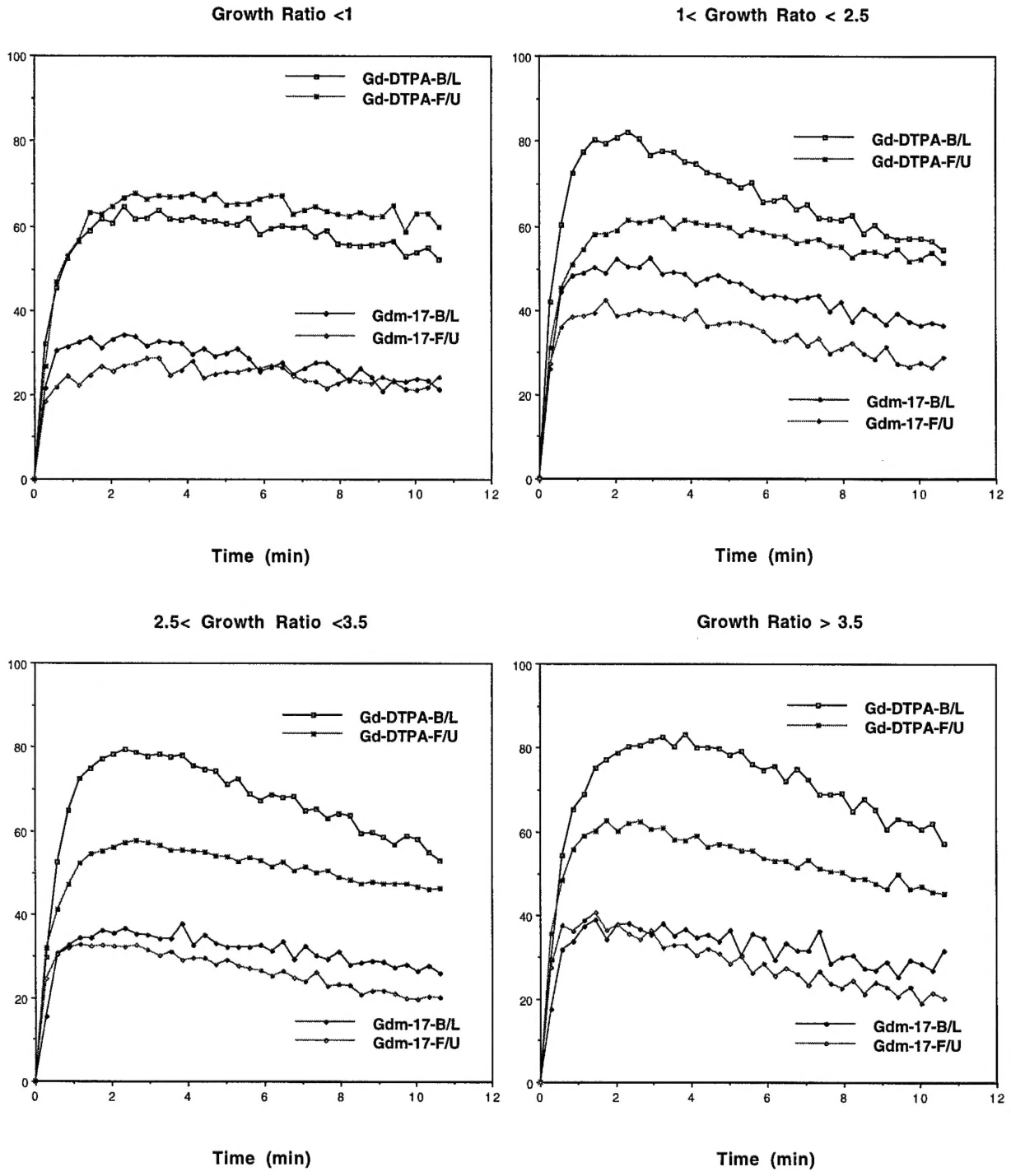

Figure 3: The enhancement kinetics of the small agent Gd-DTPA and the intermediate sized agent Gadomer-17 in the 4 groups of tumors, stratified by their different growth rates. The vertical axis shows the intensity of the signal enhancement. Group-1 had the lowest enhancement (both Gd-DTPA and Gadomer-17) in the baseline study. The Gd-DTPA enhancement was significantly lower than that in the combined Groups 3 and 4.

The enhancement kinetics measured from tumors in these 4 groups are shown in Figure 3. In Group-1 with growth ratio < 1, it has the lowest enhancement (both Gd-DTPA and Gadomer-17) in the baseline study. The patterns of enhancement in Groups 2-4 were similar. T-tests showed that Group-1 tumors which showed shrinkage had significantly lower Gd-DTPA baseline enhancements compared to that in the combined Groups 3 and 4. Although the difference compared to Group-2 was also large, it was not significant due to the large variation in Group-2. The enhancement of Gadomer-17 did not reveal significant differences, presumably due to the low enhancement, thus not sensitive to show differences.

Correlation between contrast enhancements and the percent vascular area

The enhancements measured by Gd-DTPA and Gadomer-17 in the follow-up studies, right before the tumor was excised, were correlated with the percent vascular area measured from the tumor specimens using the developed histological technique. The hot spot area which had the highest vessel density was chosen for the vascular area quantification. No significant correlation was found. The linear regression coefficient r was close to zero. One explanation for this lack of correlation might be due to that the MRI enhancement was measured from the entire tumor while the vascular area was measured from the hot spot. We will refine the technique to measure the mean vascular area from representative regions of the entire tumor, which is expected to have a better correlation with the MRI enhancements.

Correlation between the degree of inflammation with the tumor growth and the treatment

We further studied whether the inflammation was correlated with tumor growth rates. No significant correlation was found. Inflammation could not predict the growth of tumors. The tumors in each of the 4 groups with different growth rates had grade 1 and 2 inflammation. The right side of the tumor was treated with intratumoral injection of adenovirus. In each treatment group (IL1-alpha, IFN-gamma, beta-gal), the degree of inflammation between the right and left tumors was not different. In some animals the right side tumor had a higher inflammation but in others the left side tumor had a higher inflammation. Inflammation was not associated with the gene therapy treatment.

(6) Key Research Accomplishments

- Trained a research assistant for cell culture, animal work, and tissue preparation techniques to participate in this project.
- Continued to work on the experiments using magnetic resonance imaging to monitor the gene therapy induced changes.
- Worked with the researchers at Quest Diagnostic Inc. and Oncotech Inc. for developing the histological and immunohistochemical methods to stain the vessels in rat tissue, as well as assessment of inflammation.
- Developed the methods to attach MR contrast agent, Gadolinium, to the adenovirus, so that the fate and distribution of adenovirus in the host body and the cancer can be monitored using non-invasive imaging techniques.

(7) Reportable Outcomes

- One paper entitled "Prediction of Gene Therapy Induced Tumor Size Changes by the Vascularity Changes Measured Using Dynamic Contrast Enhanced MRI" has been published by "Magnetic Resonance Imaging",
- Four conference papers were presented
- Submitted a proposal entitled " Synthesis of a Detectable Neovasculature Targeted Adenovirus" to NIH, and have successfully obtained the funding (R21 CA83681).

Four Conference Abstracts:

M. Y. Su, J. Wang, J. A. Taylor, L. P. Villarreal, O. Nalcioglu, Correlation Between the Longitudinal Changes of Tumor Size and the Enhancement Kinetics in Tumors Receiving Gene Therapy. in "Proc., 9th ISMRM Annual Meeting, Glasgow, UK, 2001" p2277.

M-Y. Su, J.A. Taylor, L.P. Villarreal and O. Nalcioglu, Prediction of Gene Therapy Induced Tumor Size Changes by the Vascularity Changes Measured Using Dynamic MRI. in "Proc., 2nd Era of Hope, Department of Defense Breast Cancer Research Program Meeting, Atlanta, USA, 2000" p245.

Lydia Su; Monitoring Gene Therapy Induced Responses in Cancer by MRI. in Proceedings, Chao Family Comprehensive Cancer Center Conference, Palm Springs, October, 1999

M-Y. Su, J.A. Taylor, L.P. Villarreal and O. Nalcioglu, Prediction of Gene Therapy Induced Volumetric Changes by Intravascular Volume Changes Measured Using Dynamic Contrast Enhanced MRI. in "Proc., 7th ISMRM Annual Meeting, Philadelphia, USA, 1999" p145.

One Journal Paper:

M.-Y. Su, J. A. Taylor, L. P. Villarreal and O. Nalcioglu; Prediction of Gene Therapy-Induced Tumor Size Changes by the Vascularity Changes Measured Using Dynamic Contrast Enhanced MRI. Magnetic Resonance Imaging 18 (2000) 311-317.

(8) Conclusions

This was a career development award. Since this very first funding award to the P.I., I have successfully obtained some other grants, either serving as the P.I. or Investigators. The training experience that I acquired from this project was very important for me to become a competitive investigator. For the scientific value of this project, the achievement was not as much compared to its training value. We have demonstrated that MRI can be applied to monitor the longitudinal volumetric and vascular changes in tumors receiving gene therapy. Although the MRI results could not reliably predict the tumor growth, it can be used as a suitable monitoring modality. We further attempted to correlate the MRI findings with the underlying biological changes, including vascularity and inflammation. However, no significant correlation was found. Nevertheless, the techniques that were developed in this project can be applied to other projects in the future.

(9) Appendices

An additional conference paper that was not included in the previous reports is included.

M. Y. Su, J. Wang, J. A. Taylor, L. P. Villarreal, O. Nalcioglu, Correlation Between the Longitudinal Changes of Tumor Size and the Enhancement Kinetics in Tumors Receiving Gene Therapy. in "Proc., 9th ISMRM Annual Meeting, Glasgow, UK, 2001" p2277.

Correlation Between the Longitudinal Changes of Tumor Size and the Enhancement Kinetics in Tumors Receiving Gene Therapy

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Introduction

As new advances in immunotherapy in cancer treatment emerge, there is an increasing need to determine the efficacy of therapy as early as possible in order to optimize treatment regiments. It would be extremely useful to develop a non-invasive technique to predict treatment efficacy prior to changes in tumor growth. Previously we have reported a pilot study that applied dynamic contrast enhanced MRI to monitor the volumetric changes and the vascular changes in the C6 glioma model after receiving gene therapy [Su et al, MRI 18:311-317, 2000]. The pilot study results revealed a correlation between the tumor size changes and the vascular changes, thus suggesting that the vascular changes may be used to predict the treatment outcome. However, the pilot study results were obtained from only 6 tumors from 3 animals. We expanded the study to investigate whether the initial findings still held. Longitudinal MRI studies were applied to measure the tumor size and the enhancement kinetics before and after the gene therapy treatment. The tumors were separated into 4 groups according to their different volumetric growth patterns. The baseline enhancement kinetics and the post therapy changes in the enhancement kinetics were compared to investigate whether they could reveal distinction among them. We also investigated whether the virus treatment gave a consistent modulation in tumor's growth.

Methods

Twenty-one Wistar rats were injected with 5 x 10^6 C6 glioma cells bilaterally and subcutaneously into the rear haunch. A pre-treatment baseline MRI study was first conducted at 10-12 days after tumor implantation, when the tumor reached to approximately 0.7 cm in diameter. All experiments were performed on a 1.5 Tesla Picker Scanner using the quadrature lower extremity coil. The MRI protocol included a T2-weighted sequence covering the entire tumor for volumetric measurements and a dynamic T1-weighted sequence for contrast enhancement study. The contrast agent, Gd-DTPA (0.1 mmol/kg), was injected via the tail vein, and the kinetics were measured using a spin-echo sequence with TR= 140 ms, TE= 14 ms, FOV= 16 cm, matrix size= 256x128.

Within 24 hours after the baseline MRI study, the animals received left-sided intratumoral injection of 2 x 10^8 plaque forming units of recombinant adenovirus. We used three adenoviruses carrying mouse interferon gamma (IFN-gamma), mouse interleuken 1 alpha (IL1-alpha), or the marker gene beta-gal as the control. Eight animals received recombinant IL1-alpha, 5 animals received recombinant IFN-gamma and 8 animals received recombinant beta-gal. The post-treatment MRI experiments were repeated at 4, 7, 11, 14, and 20 days after the baseline studies. For each tumor the longitudinal volumetric changes and the changes of the enhancement kinetics were measured. According to the different volumetric growth patterns of all 42 tumors, they were stratified into four groups. Group-A tumors exhibited a continuous growing pattern, Group-B tumors grew bigger at Day-4 and even bigger at Day-7 then regressed; Group-C tumors grew bigger at Day-4, and started to regress at Day-7; Group-D tumors displayed a continuous regressing pattern. The baseline kinetics and the changes in the kinetics after treatment were compared among the 4 groups.

Results

For each tumor, we recorded the volumetric growth pattern, the baseline enhancement kinetics, and the changes of the kinetics after the treatment. Four groups of tumors were separated according to their growth patterns. Figure 1 shows the longitudinal volumetric growth pattern in groups B-D. Group-B tumors (n=8) grew bigger at Day-4 and even bigger at Day-7. Group-C tumors (n=13) grew bigger at Day-4 and started to regress at Day-7, and Group-D tumors (n=17) regressed at Day 4 and further at Day-7. All of them exhibited a continuous regression after day 7. The other 4 tumors, assigned to group-A exhibited a continuous growing pattern, not included in the figure here. The virus treatment to the tumor at the treated side or the contralateral site did not cause a consistent modulation in their growth pattern. The tumors received the recombinant IL1-alpha, IFN-gamma, or beta-gal were distributed in all 4 groups.

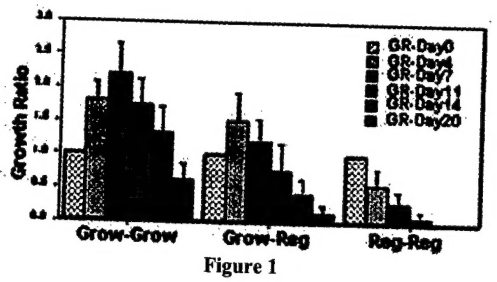

Figure 1

Figure 1. The growth pattern (from Day-0, 4, 7, 11, 14 to 20) of the 3 groups of tumors stratified by their size changes at Day-4 and Day-7. Group B: Grow-Grow, Group C: Grow-Regress, Group D: Regress-Regress.

We were interested in studying whether the baseline enhancement kinetics or the changes in the kinetics can predict their future growth pattern as demonstrated in the pilot study. Figures 2a and 2b show the enhancement kinetics measured from the tumors in Groups A and D. The baseline kinetics from these 2 groups had a comparable magnitude, although a decay phase could be noted in Group-A, but not D. At Day-4 the tumors in group-A grew larger and those in Group-D regressed, but the enhancement kinetics were very similar; they didn't exhibit much change compared to the baseline curve. In all 4 groups, neither the baseline kinetics or the changes in the longitudinal study revealed significant differences among them.

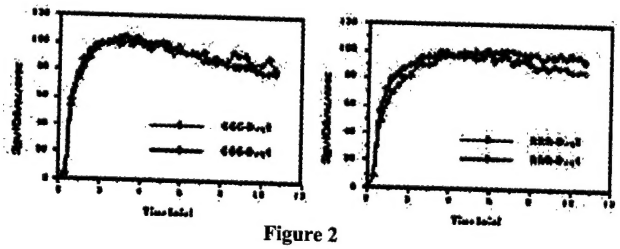

Figure 2

Figure 2. The mean enhancement kinetics from tumors in Group-A which exhibited a continuous growing pattern (a), and the mean enhancement kinetics from the tumors in Group-D which exhibited a continuous regressing pattern (b). Day-0 kinetics is shown by filled symbol, and Day-4 kinetics is shown by open symbol.

Discussion

MR imaging is a non-invasive technique that can been used to assess the change of tumor size accurately. Dynamic contrast enhanced MRI can be further applied to measure the enhancement kinetics from the tumor, then from which the vascular parameters can be derived. In a pilot study we demonstrated that the changes of vascular volume, determined using dynamic contrast-enhanced MRI, is predictive of future tumor growth. However, the finding did not hold up in this expanded study. Gene therapy induces a rather complicated immuno response. That does not seem to affect the vasculature as would be the case with anti-angiogenic therapy. Our results suggest that the changes in the enhancement kinetics measured by dynamic contrast enhanced MRI may not be useful to reflect or even predict the treatment outcome of gene therapy.

Acknowledgement

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